Field of the invention

The invention pertains to the use of TRPM8-modified substances for the production of pharmaceutical composition for the treatment of tumor diseases. The invention also concertns such compositions as well as a treatment plan.

Background of the invention and state of the art

In the following the terms Trpp8 and TRPM8 are used synonymously.

Calcium homeostasis regulates important cell functions such as proliferation, differentiation, invasion, migration, angiogenesis and apoptosis. In prostate cancer calcium plays an important part in tumor formation. However, little is known of the calcium channels and membrane-bound plasma receptors that regulate the entering and exiting of calcium into and out of intracellular calcium reservoirs in prostate tumor cells.

Tripp8 has been described in the report by Tsavaler et al., Cancer Res. 61:3760-3769 (2001) as a prostate-specific gene that is expressed predominantly in human prostate tumors. Trpp8 is significantly up-regulated. Trpp8 is found in androgen-dependent prostate cell lines accord to this source but not in androgen-independent cell lines which also do not express PAP (prostate acid phosphatase) and PSA (prostate-specific antigen). It is theorized that Trpp8 functions as a calcium channel protein.

Trp proteins are said to belong to the so-called store operated calcium channels (SOC) or capacitative calcium entry channels (CCE). An involvement in apoptosis could be demonstrated in LNCaP cells (Wertz et al., J. Biol. Chem. 275:11470-11477 (2000)).

The 5694 bp Trpp8 cDNA has a 3312 bp open reading frame which codes for an 1104 amino acid protein with purportedly seven transmembrane domains with a molecular weight of ca. 137,500 Da.

Trpp8 Sequences are described in the literature sources US-6,194,152, US-6,183,968, WO-99/46374, WO-99/09166, WO-01/25273, WO-01/25272, WO-01/34802, WO-01/46258, WO-01/42467 and WO-01/1633. The literature sources US-6,194,152 and WO-01/51633

disclose the usage of the sequences therein named for the detection of tumor cells and of different classes of subtances generally for the treatment of prostate cancer.

Menthol is a secondary plant substance which occurs naturally as the monoterpene in peppermint and comprises the main constituent of peppermint oil. Menthol induces a cold sensation on the skin and in the mouth and nose by exciting certain nerve cells. Icilin is another substance causing a cold sensation. Both of these substances activate peripheral nerve cells, whereupon the TRPM8 ion channel is selectively activated, and ions such as Ca2+ and Na+ can flow into the cell. From the literature sources McKemy et al., Nature 416(6876):52-52 (2002) and Feier et al., Cell 108 (5) :705-715 (2002) it is known that the human-orthologous TRPM8 functions as a menthol sensor in mice and rats. The same is known for icilin. TRPM8 also functions as a cold receptor in a temperature range from 8 to 25°C.

No physiological function of TRPM8 in tumor tissues is known.

Prostate cancer, in particular, is a disease that occurs with considerable incidence with increasing age. Heretofore prostate cancer has essentially been diagnosed pathologically and is usually treated by removal of the prostate. The removal of the prostate has various unfavorable effects on a patient. An improved diagnosis and treatment of this form of cancer, especially without the necessity of removing the prostate, is therefore highly desirable.

Technical problem of the invention

The invention addresses the technical problem of obtaining pharmaceutical compositions for treatment of tumor diseases, especially prostate cancer.

Basic features of the invention and preferred examples of embodiment

To solve this technical problem the invention teaches the use of a TRPM8-activating substance to produce a pharmaceutical composition for the treatment of tumor diseases, especially of prostate cancer, in which TRPM8 is overexpressed.

The invention is based on the surprising discovery that the activation of TRMP8 inhibits and/or retards the growth of tumors displaying an elevated expression of the TRPM8 ion channel. In particular a permanent activation specifically destabilizes the ion balance of the tumor cells which are driven into apoptosis as a result.

A substance is preferentially used that is selected from the group consisting of "menthol, menthyl derivatives, pyrrolidinyl derivatives of furanone, icilin, icilin derivatives and mixtures of these substances." The term 'menthol' includes all enantiomers as well as mixtures of the enantiomers. The same is true for other substances or substance classes named which have symmetry centers. Furthermore, substances structurally different from the substances named above may be used, the activation of TRPM8 being regarded as the essential selection criterion. 2-Isopropyl-N-2, 3-trimethylbutyramide is an example of one such different substance.

Menthyl derivatives may be constructed in particular according to formula I, in which ... may be a single or double bond, ... may denote a single bond or no bond, wherein the not-shown valences of carbon are saturated with –H, where R1 = -H, -OH, -SH, -NR11R12, C1-C10-alkyl, -aralkyl or -aryl, for example, methyl or ethyl, where R11 and R12 may be the same or different and -H, C1 to C10-alkyl, -aralkyl or -aryl, where R2 may be = -OR21, -SR21, -CO-R22, or -O-CO-R23, where R21 may be = -H, C1-C10-alkyl, -aralkyl, -aryl, or C1-C10-alkylpolyethers with l to 5 ether groups, non-, mono- or poly-substituiert, especially -OH or -SH substituted, where R22 may be = -H, Cl-ClO-alkyl, -aralkyl, -aryl, or C1-C10-alkylpolyethers with l to 5 ether groups, non-, mono- or poly-substituted, especially -OH or -SH substituted, or may be -NR221R222, where R221 and R222 may be the same or different and may be -H, C1 to C10-alkyl, -aralkyl, -aryl, or C1-C10-alkylpolyethers with l to 5 ether groups, where R23 may be = -H, C1-C10-alkyl, -aralkyl, -aryl, or C1-C10-alkyl polyethers with l to 5 ether groups, non-, mono- or poly-substituted, especially –OH or –SH substituted. Examples of menthyl derivatives are: Isopulegol (... = double bond, ... = no bond, R2 = -OH), menthoxypropane-1,2-diol (... = single bond, ... = single bond, Rl = -H, R2 = -O-CH2-CHOH-CH2-CH2OH (N-ethyl-p-menthane-3-carboxamide) (... = single bond, ... = single bond, Rl = -H, R2 = -CO-NH-CH2-CH3) and p-menthane-3,8-diol (... = single bond, ... = single bond, Rl = -OH, R2 = -OH). Other examples are 3-menthyl-3,6-dioxaheptanoate, 3-menthylmethoxyacetate, 3-menthyl-3,6,9-trioxadecanoate, 3-menthyl(2-hydroxyethoxy)acetate and menthyl-l1-hydroxy-3, 6, 9-trioxaundecanoate (... = single bond, ... = single bond, Rl = -H, R2 =C1-C10-alkyl polyether with l to 5 ether groups, not or -OH substituted). Another example is menthyl lactate (... = single bond, ... = single bond, Rl = -H, R2 = -O-CO-R23 and R23 = hydroxyethyl).

Pyrrolidinyl derivatives of furanone may be constructed especially according to formula II, where R1 and R2 are present at least singly, in which case R1 and R2 may be bound to every free carbon valence of the furanone ring, the free carbon valences being saturated by hydrogen, especially by C1-C10-alkyl, -aralkyl, -aryl, -OH or –NH2, while pyrrolidine is preferably bound via N to the furanone ring, in which case R2 may be = C1-C10-alkyl, -aralkyl, -aryl, -OH, -NH2 and where R2 is present preferably singly or doubly and where R1 is preferably present singly. Examples are: 5-Methyl-4-(1-pyrrolidinyl}-3-[2H]-furanone, 4, 5-dimethyl-3-(1-pyrrolidinyl)-2-[5H]-furanone, 4-methyl-3-(1-pyrrolidinyl)-2-[5H]-furanone.

Icilin is represented in formula III. Also included are icilin derivatives which activate TRPM8. This can be tested without difficulty according to the examples of embodiment. A common feature of all substances named is the fact that they trigger cold sensations upon contact with the skin or mucous membranes.

A pharmaceutical composition according to the invention can be prepared galenically with conventional accessory and carrier materials in the customary way, preferably for injection, i.v., i.p., or i.m. or infusion. The dose is preferably adjusted in the range from 0.1 to 5000 mg/kg body weight, preferably 1 to 100 mg/kg body weight, relative to one day, divisible into 1 to 10 dosage units. It is advisable to prepare the composition for continuous or discontinuous periodical administration over a time interval of at least 2 weeks, preferably at least 8 weeks, most preferably at least 20 weeks. This is to be linked to a treatment plan which envisions continuing administration in these time intervals. A discontinuous periodical administration is accomplished by giving a single dose at specified times. The time intervals may be, for instance, in the range of 1 hour to 7 days. Continuous adminstration is achieved with suitable systems causing a continuous release of the substance. For example, therapeutic substances adsorbed on or in polymeric microparticles come under consideration, with which the substances are released slowly from the injected microparticles. Such systems are well known in numerous variants to the average man of the art. The systems continuously releasing active principles also include transdermal systems which are also familiar in numerous variants to the average man of the art.

Finally, the invention also discloses a process for the treatment of tumor diseases, especially prostate cancer, by administering a physiologically active dose of a TRPM8-inhibitor to a patient with the disease.

It is possible within the scope of the invention to use the pharmaceutical compositions according to the invention in combination with local hypothermia, in which case the tissue to be treated is preferably cooled to a temperature below 36°C, especially below 30°C, preferably below 25°C. The hypothermia may be continuous or discontinuous. In the case of discontinuous hypothermia it may be applied before, during and/or after the administration of the pharmaceutical compositions of the invention.

Definitions:

In this description the term TRPM8 is used for all human iso-forms, known or new, based on amino acids. In this description TRPM8 is also called Trpp8. In particular, the proteins and peptides coded by the nucleic acids disclosed in the sequence listings as well as the proteins and peptides disclosed in the sequence listings are included, just as are the TRPM8 sequences and the proteins or peptides coded by them which are disclosed in the cited reference works. This term also encompasses the short sequences disclosed in this description that stem from the iso-forms, e.g., immunization sequences. Also included are homologs, in which case the homology amounts to at least 80%, preferably more than 90%, most preferably more than 95%, as calculated by the BLAST program in the version current on the date of filing of the application. Also included are sequences that represent only partial sequences of the explicitly disclosed sequences, e.g., an exon or several exons or sequences complementary to them, with the qualification that the latter bind to a protein or peptide-specific target molecule with at least the same affinity, especially the substances used according to the invention.

In connection with applications according to the invention, the definitions of the proteins and peptides include, besides the full lengths of the disclosed sequences (see also preceding paragraph) also partial sequences from them with an average length of 4 amino acids, preferably 10 to 30 amino acids.

The definition of treatment also includes prophylaxis.

A tumor cell overexpresses TRPM8 if the quantity of formed TRPM8 RNA or formed TRPM8 protein in a tumor cell is greater than in normal cells of the same tissue type, preferably

obtained from the same patient. It is to be understood that the same measurement processes are used for the tumor/normal comparison. The man of the art is familiar with various measurement processes for determining nucleic acids and/or proteins and peptides in cells, all of which are applicable.

A compound or substance is called an activator if it either promotes the formation of TRPM8 or raises the activity of formed TRPM8 relative to the TRPM8 activity in the absence of the activator. A substance may therefore be an activator, on the one hand, if it intervenes in an activating way in the TRPM8 formation cascade. On the other hand, a activator may be a substance that forms a bond with formed TRPM8 in such a way that further physiological interactions with endogenous substances are increased compared with the same interactions without binding of the activator. An activator increases preferably upon contact with cells expressing TRPM8 the transport of ions into a cell or out of it compared to a cell with the same TRPM8 expression level but without contact with the activator. The ion transport can be determined, e.g., according to the article by Peier et al., Cell 108(5); 705-715 (2002).

A pharmaceutical composition according to the invention can be prepared galenically in the customary manner. Na+, K+ or cyclohexylammonium may be considered as antions for ionic compounds. Suitable solid or liquid galenic forms are, for instances, granulates, powders, pills, tablets, (micro)capsules, suppositories, syrups, juices, suspensions, emulsions, drops or injectable solutions (i.v., i.p., i.m.) and preparations with prolonged release of the active principle, in the production of which conventional accessories such as carriers, bursting, binding, coating, swelling, sliding agents or lubricants, flavoring substances, sweeteners and solution promoters may be used. As accessories one may mention magnesium carbonate, titanium dioxide, lactose, mannitol and other sugars, talcum, milk protein, gelatins, starch, cellulose and its derivatives, animal and vegetable oils such as cod-liver oil, sunflower seed oil, peanut or sesame oil, polyethyleneglycols and solvents such as sterile water and monovalent or polyvalent alcohols, such as glycerine. A pharmaceutical composition according to the invention can be produced by mixing at least one TRPM8 activator used according to the invention in a defined dose with a pharmaceutically suitable and physiologically tolerable carrier and optionally other suitable active, additional or accessory substances with defined doses and fabricating them into the desired form for administration.

The concepts/definitions expanded with respect to the narrow meaning of the words in the definition above also include the concepts as defined by the strict or narrow meaning of the words. Statements regarding a category of claims as well as claims dependent on an independent claim are similarly applicable to claims of a different category.

Examples of embodiment:

Example 1: Reducing the colony formation rate.

HEK293 cells were not transfected, transfected with TRPM8 or transfected with an empty vector. The cells were used in a soft agar assay (see reference Shappel et al., Cancer Research 61:497-503 (2001)). The cells highly individually plated out and immobilized in agar are caused to grow three-dimensionally and independently of the substrate in this way. The colony formation rate permits conclusions regarding the tumorigenicity of the cells to be drawn. 1000 cells were plated out in the 6-hole plate in 2 ml of medium containing soft agar and coated with 1 ml of medium (DMEM with 10% FCS, 2mM glutamine) after the agar congealed. Menthol dissolved in ethanol was added to the medium in end concentrations of 10, 100, and 100 μM and substituted every fifth day. The solvent alone was added as a control. After three weeks the number of colonies formed was determined under the microscope. The TRPM8 transfected cells display distincly less colony formation than the wild type cells and the cells transfected with the empty vector.

Example 2: Tumor growth in hairless mice.

Human TRPM8 cDNA was subcloned in the expression vector pcDNA3.1 and subsequently stably transfected in HEK293 cells. The expression of TRPM8 protein was demonstrated in the Western Blot with TRPM8-specific antibodies. To study the effect of menthol or icilin on tumor growth in vivo 2 million HEK293 TRPM8 cells were subcutaneously injected or xenotransplanted in the prostate in male hairless mice. The test groups in each case consisted of 10 animals. The control groups were untreated or treated only with DMSO. The animals were treated by daily intraperitoneal administration of 20 mg/kg body weight icilin or menthol, dissolved in DMSO, over a time interval of three weeks. The growth of the subcutaneously injected cells was measured twice weekly over the entire test duration.

Immediately after completion of the trials the "xenotransplants" were resected, weighed and preserved. The result was that the treated animals displayed distinctly less tumor growth than the untreated control animals.

Example 3: TRPM8 sequences

TRPM8 sequences, especially splice variants, are listed in the sequence protocols. In the case of the nucleic acid sequences the latter code for proteins, peptides or partial sequences of proteins or peptides that may be activated within the scope of the invention. Amino acids pertain to sequences, proteins, peptides or partial sequences of proteins or peptides capable of being activated within the scope of the invention. Additional sequences for TRPM8 may be obtained from the references cited above.

Supplement Page 2 Background of the invention

Neuroendocrine tumors (NET), previously also called carcinoid tumors, are potentially malignant tumors developing out of hormone-producing (endocrine) cells. NET of the gastrointestinal tract are also called gastro-entero-pancreatic (GEP) tumors. NET may also occur in organs of the respiratory tract, e.g., the bronchi or the lungs. Little is know of the potassium homeostasis of these rare cancers, especially the part played by TRP channels in NET.

Supplement Page 10 Definitions

Nanosuspension (nanocrystals in aqueous solution smaller than 1 μm).

Supplement Page 14 Claims

Use as in Claim 1, wherein the tumor disease is neuroendocrine tumors, especially of the gastrointestinal tract and respiratory organs.

Replacement of Fomulas I-III with new drawings

Supplement Examples of embodiment

Example 1 Reduction of the colony formation rate becomes Example 5

Example 2 Tumor growth in hairless mice becomes Example 6.

Example 3: TRPM8 sequences becomes Example 10.

Example 1: Icilin induces cytotoxicity in TRPM8 transfectants

HEK293 cells were stably transfected with TRPM8 (K52) or stably transfected with empty vector (M2) 5000 cells were plated out in each 96 well plate in 100 μl medium and the next day were mixed with icilin dissolved in DMSO in the end concentrations of 30 μM, 10 μM and 2 μM. Completely untreated cells (Ko) and cells that were mixed with DMSO in a dilution corresponding to the highest icilin concentration served as controls. After 48 h of incubation the cells were photographed under the microscope. A distinct concentration-dependent cytotoxic effect by icilin on HEK293 TRPM8-transfectands was noted, but no such effect on the control cells. The cytotoxicity correlates with a dramatic change in cell morphology.

DMSO had no effect on cell growth or cell morphology.

Example 2: Icilin has an antiproliferatory effect on TRPM8 transfectands.

HEK293 cells were stably transfected with TRPM8 (K52) or stably transfected with empty vector (M2). 5000 cells were plated out in each 96well plate as a 6-fold batch in 100 μl

medium and the next day mixed with icilin dissolved in DMSO in the end concentrations of 10 μM 5 μM, 1μM and 100 nM Completely untreated cells (Ko) and cells that were mixed with DMSO in a dilution corresponding to the highest icilin concentration served as controls. After 48 h of incubation the cell proliferation was determined by luminometric quantification of the intracellular ATP concentration. The relative light units (RLU) are shown in relation to the untreated control cells. The presence of icilin causes a distinct concentration-dependent inhibition of proliferation in TRPM8-positive cells, while no effect on control cells was observed. Similar results were observed with other proliferation assays, e.g., MTS, MIT, and XTT.

Example 3: Icilin has a pro-apoptotic effect on TRPM8 transfectands.

HEK293 cells were stably transfected with TRPM8 (K52 or stably transfected with empty vector (M2).5000 cells were plated out in each 96well plate as a 6-fold batch in 100 μl medium and the next day mixed with icilin dissolved in DMSO in the end concentrations of 10 μM, 5 μM, 1 μM and 100 nM. Completely untreated cells (Ko) and cells that were mixed with DMSO in a dilution corresponding to the highest icilin concentration served as controls. After 24 h of incubation the apoptosis induction was determined by fluorometric quantification and Caspase3/7 activity. The relative light units (RLU) in relation to untreated controls are shown. The presence of icilin causes a distinct concentration-dependent apoptosis induction in TRPM8-positive cells, while no effect on control cells was observed. Similar results were obtained with other apoptosis assays, e.g. PARP-Western Blot.

Example 4: Icilin has an antiproliferatory effect on LNCaP cells.

8000 cells of the prostate tumor cell line LNCaP were plated out in each 96well plate as a 6-fold batch in 100 μl medium and the next day mixed with icilin dissolved in DMSO in the end concentrations of 3 μM. In addition, Paclitaxel (Pax) in a concentration of 10 nM and in combination with icilin in the above-reported concentration were used. Cells that were mixed with DMSO in a dilution corresponding to the highest icilin concentration served as controls. After 48 h of incubation the cell proliferation was determined by luminometric quantification of the intracellular ATP concentration.The relative light units (RLU) in relation to the untreated control cells are represented. The presence of icilin in LNCaP cells which express TRPM8 endogenously causes a distinct concentration-dependent inhibition of proliferation, while no

effect on control cells was observed. Paclitaxel also has a proliferation-inhibiting effect. The combinatin of icilin with Paclitaxel has a stronger proliferation-inhibiting effect than the two substances alone (synergistic effect). Similar results were observed with other proliferation assays, e.g., MTS, MIT, and XTT.

Example 5: Icilin reduces the colony formation rate.

Stably TRPM8-transfected HEK293 cells (K51, K52) were immobilized in soft agar. The colony formation rate was determined as a measure of the substrate- independent growth. 1000 cells were plated out in 2 ml medium in the 6-hole plate. The addition of icilin in the end concentrations of 1 μM and 100 μM and the solvent control DMSO (Ko) corresponding to the highest icilin concentration was substituted in each case after 48 h in the supernatant. The supernatant (2 ml) was always replaced after 96 h. After a total of 14 days the colonies grown in the agar were stained with Neutral Red, dried on cellulose, and photographed. The addition of icilin causes a distinct concentration-dependent inhibition of the number of living colonies.

Example 6: Icilin reduces tumor growth in hairless mice.

Stably TRPM8-transfected HEK293 cells (K52) were xenotransplanted intraperitoneally (i.p.) into hairless mice (NMRI nu/nu, 9 weeks old, male, 2 million cells per animal). The animals were treated i.p. every 3rd day over a time of 14 days with 20 μl of a 100 mM icilin solution in DMSO. The control group was treated with DMSO alone under identical conditions. The tumor growth was monitored by daily determination of the body weight. The icilin treatment caused a distinctly reduced tumor growth compared to the solvent-treated control group.

Example 7: TRPM8 is express in neuroendocrine tumors.

A) From a lung adenocarcinoma and two lung tumors with neuroendocrine differentiation tumor [tissue] and corresponding normal epithelial tissue were excised. The mRNA was prepared and the TRPM8 expression quantified by RT-PCR analysis. The relative expression of tumor versus normal epithelial tissue is shown. The tumors with neuroendocrine differentiation display a distinct TRPM8 expression, while as opposed to this no relevant TRPM8 expression is present in the adenocarcinoma.

B) The mRNA was prepared from human neuroendocrine tumor cell lines obtained from pancreas carcinoma (BON-1, QGP-1) and colon carcinoma (LCC-18), and the TRPM8 expression quantified by RT-PCR analysis. The relative expression of the mRNA compared with

TRPM8-positive LNCaP prostate tumor cells. All three neuroendocrine tumor cell lines tested express TRPM8 in significant quantities.

Example 8: Icilin has a pro-apoptotic effect on neuroendocrine tumor cells

Human neuroendocrine QGP-1 pancreas tumor cells were plated out in 96well plates in 100 µl medium (5000 cells/well) and the next day mixed with icilin dissolved in DMSO in the end concentrations of 100 nM 1 µM, 10 µM and 100 µM. Completely untreated cells (Ko) and cells that were mixed with DMSO in a dilution corresponding to the highest icilin concentration served as controls The presence of icilin causes a distinct concentration-dependent apoptosis induction in QGP-1 cells, while scarcely any effect on control cells was observed. After 24 h of incubation the apoptosis induction was determined by fluorometric quantification of the Caspase3/7 activity. The presence of icilin caused a distinct concentration-dependent induction of apoptosis in QGP-1 cells while only minor effects were observed in control cells.

Example 9. Icilin has an anti-proliferatory effect on neuroendocrine tumor cells.

Human neuroendocrine QGP-1 pancreas tumor cells were plated out in 96well plates in 100 µl medium (5000 cells/well) and the next day mixed with icilin dissolved in DMSO in the end concentrations of 100 nM 1 µM, 10 µM and 100 µM. Completely untreated cells (Ko) and cells that were mixed with DMSO in a dilution corresponding to the highest icilin concentration served as controls. After 48 h of incubation the cell proliferation was determined by luminometric quantification of the intracellular ATP concentration. The proliferation rate is shown in relation to the solvent-treated control cells. The presence of icilin causes a distinct concentration-dependent inhibition of proliferation in QGP-1 cells, while no effect on control cells was observed. Similar results were observed with other proliferation assays, e.g., MTS, MIT, and XTT.